

HYPERBARIC OXYGEN AND EMBRYONIC DEVELOPMENT IN *ARBACIA PUNCTULATA*¹

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Oxygen toxicity to living systems has been recognized since the classical studies of Bert (1878), but relatively little attention has been directed toward determining the effects of oxygen at high pressure upon developing systems. Such studies do include those of New and Coppola, 1970 (rat); Pizzarello and Shircliffe, 1967 (chick); Ferm, 1964 (hamster); Rosenbaum, 1960, Malamed, 1957, Nelson, 1949, and Rauber, 1884 (frog); and Rosenbaum and Wittner, 1960 (sand dollar). Miller, Miller, DeSha and Heidger (1969) investigated the effects of hyperbaric oxygen (HBO) upon embryonic development in the hydroid, *Tubularia*, and demonstrated that differentiation is blocked by exposure to pure oxygen at pressures of 2 to 4 atmospheres absolute (AA). Blockage of differentiation was accompanied by inhibition of succinic dehydrogenase activity. In view of the high succinic dehydrogenase activity which prevails during portions of sea urchin development (Gustafson and Hasselberg, 1951), it was of interest to extend our investigation to another phylum, the Echinodermata, and to a species from which large numbers of embryos are readily obtained and are routinely reared *in vitro*. This paper presents the results of studies of the effects of hyperbaric oxygen upon the embryonic development of *Arbacia* from fertilization to the time of formation of the pluteus larva.

MATERIALS AND METHODS

Specimens of *Arbacia punctulata* collected by the Supply Department of the Marine Biological Laboratory were used in all experiments. Gametes were shed by electrical stimulation (Harvey, 1956), and the eggs, pooled from several females, were suspended in filtered sea water at room temperature and were washed several times. They were inseminated with a dilute sperm suspension. The percentage fertilization was assessed 10 minutes post-insemination by observation of the elevation of the fertilization membrane, and all samples showing less than 95% fertilization were discarded.

The fertilized eggs were then distributed to finger bowls or Petri dishes such that a single layer of zygotes was distributed over the bottom of the container. The zygotes then were transferred to a 16° C constant temperature room, and were assigned randomly to control or experimental groups. Control groups were allowed to develop in air at 16° C, and experimental groups were subjected at the

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same temperature to 100% oxygen at 3 AA, within a Bethlehem hyperbaric chamber. Compression and decompression of the chamber were performed slowly such that no temperature changes occurred. The chamber was thoroughly flushed with gas before each compression. All times reported in this study were recorded from the time of insemination, taken to correspond closely with the time of fertilization.

Sampling

Sampling was accomplished by two means. In the case of a continuing culture of embryos, an aliquot of embryos and sea water was withdrawn by pipette after the entire culture had been agitated by a stream of air. In the case of terminal samples, the entire culture was fixed by the addition of several ml of 5% glutaraldehyde in sea water after a sample had been removed for assessment of viability. Two investigators routinely checked the samples by the double blind technique. Staging of embryos was carried out according to Harvey, 1956.

Histological studies

Control and experimental embryos were fixed by immersion in 3% glutaraldehyde in sea water for 24 hours. The embryos were washed in filtered sea water, dehydrated in alcohols, cleared in xylene, and embedded in Tissuemat (Fisher). Seven-micron sections were cut and were stained with Ehrlich hematoxylin and alcoholic eosin-Y. Both embryos and histologic preparations were examined and photographed using a Reichert Zetopan photomicroscope.

Three principal series of experiments were performed: *Series 1*, To determine the effect upon development of constant exposure to hyperbaric oxygen. Embryos were exposed to 3 atmospheres absolute oxygen for 72 hours, post-fertilization age. Controls developed in air at normobaric pressure. *Series 2*, To determine the reversibility of effects found in Series 1 experiments. Embryos were exposed to 3 atmospheres absolute oxygen for 12, 24, 36 or 48 hours. They were removed from the hyperbaric chamber and were then allowed to develop in air for up to 144 hours post-fertilization age. Controls developed in air at normobaric pressure. *Series 3*, To determine the time during development at which hyperbaric oxygen first exerts its effect. Embryos were allowed to develop in air for 12, 18, 24, or 36 hours and were then subjected to hyperbaric oxygen until 72 hours, post-fertilization age. Controls developed in air at normobaric pressure.

Special controls

In addition to the controls outlined above, two series of controls were performed for the following reasons.

Control series 1. To exclude the possibility that observed results might be caused by exposure to 100% oxygen (Linde), and not by oxygen under pressure, embryos were incubated under 1 AA pure oxygen for 72 hours.

Control series 2. To exclude the possibility that observed results might be caused by increased pressure, and not to oxygen under pressure, embryos were incubated in the hyperbaric chamber under 1 AA pure oxygen, and 2 AA pure nitrogen for 72 hours.

RESULTS

The key developmental stages and the time at which each was observed during development at 16° C are as follows: early blastula, 6 hours; hatching blastula, 12 hours; mesenchyme blastula, 24 hours; gastrula, 30 hours; late gastrula-early prism, 36 hours; and young plutei, 48 hours (see Figs. 9, 10 and 11).

Series 1 experiments

The results of this series are summarized in Table I. Both control and hyperbaric groups developed in synchrony to the gastrula stage of development. At the time of late gastrula, however, the hyperbaric embryos appeared arrested and did not proceed to form prisms or plutei. Rather, by 48 hours, most of the embryos had lost the archenteron structure distinguishing the gastrula, and were,

TABLE I
*The effect upon Arbacia development of exposure to 3 A.A. oxygen
for various lengths of time*

Length of exposure to 3 AA O ₂	Treatment	Per cent of sample*						
		Dead or abnormal	Unhatched blastula	Mesenchyme blastula	Normal gastrula	Abnormal gastrula or regressing gastrula	Prism	Pluteus
12 hr	Control	6	94					
	Hyperbaric	5	95					
24 hr	Control	6	0	94				
	Hyperbaric	7	0	93				
36 hr	Control	6	0	0	94			
	Hyperbaric	6	0	0	80	14		
48 hr	Control	4	0	0	2	0	7	87
	Hyperbaric	5	0	0	1	94	0	0

* Minimum of 500 embryos counted in each sample.

as assessed by optical section of whole embryos, "regressing" to a configuration reminiscent of the blastula stage, but with a loose, disorganized population of cells within the central lumen. This impression which was gained from the examination of whole embryos (see Figs. 4 and 5), was verified by histological preparations (see Figs. 7 and 8). These figures illustrate that the relationships of the peripheral cells of the embryo appear to have been maintained, but that the archenteron structure was disorganized and was not identifiable as such. It is noteworthy that in each of the samples studied in this series both control and hyperbaric groups contained comparable numbers of dead or unclassifiable embryos. Therefore, HBO did not exert an immediate lethal effect, but produced a specific alteration in morphogenetic pattern. However, if the embryos were maintained in HBO continuously for 72 hours, the embryos disaggregated and died.

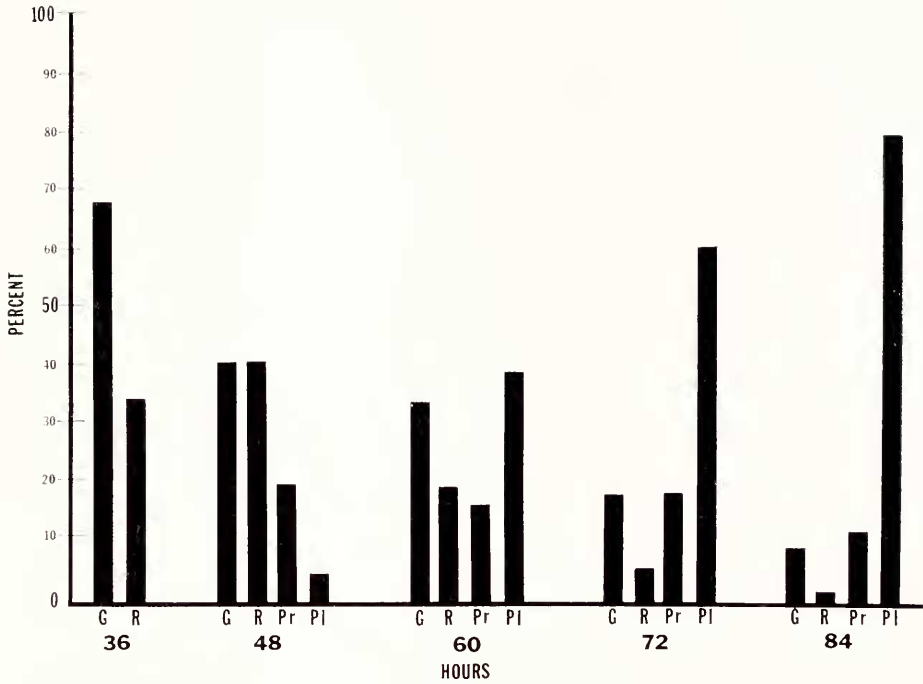


FIGURE 1. Bar graphs showing the per cent of embryos at given developmental stages at various times following removal of the embryos from 3 AA oxygen at 36 hours post-fertilization age. Abbreviations are: G = gastrula; R—regressing gastrula; Pr—prism stage; Pl—pluteus. A minimum of 200 embryos was counted at each age.

Series 2 experiments

Embryos were exposed to HBO for 12, 24, 36 or 48 hours and were then removed to air and subsequent development was observed.

(a) *Embryos removed at 12 or 24 hours.* Embryos exposed to HBO during the first 12 hours of development only were not altered morphologically, and developed at the same rate as did controls. Development was retarded in embryos exposed to HBO for the first 24 hours of development only, retardation being evidenced between the time of removal (mesenchyme blastula stage) and pluteus formation. No abnormal morphological alterations were detected, however. By 60 hours, nearly all such embryos had reached young pluteus stage. This represents a delay of 12 hours beyond the time at which all control embryos reached this stage (48 hr).

(b) *Embryos removed at 36 hours.* (See Fig. 1) At the time of removal, the embryo population consisted almost entirely of morphologically normal gastrulae, plus blastula-like forms designated "regressing gastrulae." No prism stages were seen; by 48 hours following removal, 17% of the population consisted of prisms, and 3% of normal young plutei. The relative numbers of prism and pluteus stages which were seen increased through 84 hours, when the experiment was terminated with over 90% of the embryos beyond gastrulation stage, *i.e.*, in prism or pluteus

stages. The per cent of dead or unclassifiable embryos at all times studied was never more than 6% of the total population, and is not separately plotted in the text figures.

(c) *Embryos removed at 48 hours.* (See Fig. 2) At the time of removal from HBO, 37% of the embryo population was classified as regressing gastrula. A large proportion of the embryos classified as gastrulae were not normal gastrulae, but showed varying degrees of internal disorganization. For purposes of uniformity of classification, however, any embryo showing any evidence whatsoever of archenteron structure was placed in the gastrula category. Twenty-four hours following removal, a small population (2%) of prism stages was seen. Between 72 and 96 hours, plutei were formed, and the prism population increased to 11%. The appearance of the later stages of development was accompanied by a decrease in the regressing gastrula population from 41% at 72 hours to 17% at 96 hours. Gastrulae at this time appeared normal. The decline in gastrulae and regressing gastrulae and the increase in numbers of later embryonic stages continued, until at 144 hours, 94% of the population had progressed past the gastrula stage into prisms or plutei.

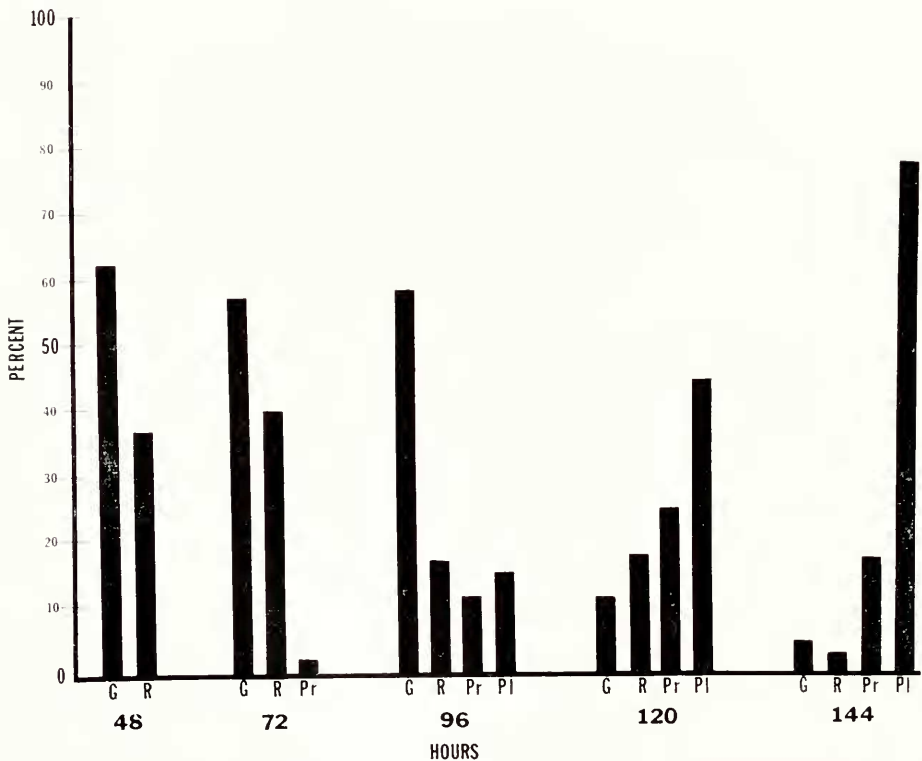


FIGURE 2. Bar graphs showing the per cent of embryos at given developmental stages at various times following removal of the embryos from 3 AA oxygen at 48 hours post-fertilization age. Abbreviations as in Figure 1. A minimum of 200 embryos was counted at each age.

Series 3 experiments

Table II summarizes the per cent of embryos reaching developmental stages 72 hours post-fertilization when exposed to HBO at various times in development. Embryos exposed following 12 hours of development in air (hatching blastula stage) gastrulated at the same time as did controls, but formed the blastula-like structures termed "regressing gastrulae" and showed early evidence of disaggregating by 72 hours. Unlike the regressing gastrulae seen in Series 1, rudimentary spicules were occasionally observed in this group embedded within the structure of the regressing gastrulae. Embryos exposed to HBO at 18 hours (having reached the swimming blastula stage), or at 24 hours (having reached mesenchyme blastula stage) were delayed in development, but showed a low percentage of regressing gastrulae, and a high percentage of normal gastrulae, prisms, and plutei at 72 hours. Embryos exposed following 36 hours of development in air (having reached the gastrula stage) progressed uninhibited to form young plutei by 48 hours. The plutei from this group when observed appeared morphologically normal but swam very slowly

TABLE II
*The effect upon Arbacia development of exposure to 3 A.A. oxygen
at various post-fertilization ages*

Age when first exposed to 3 A.A. oxygen	Per cent of embryos in stages at 72 hours of age*				
	Dead	gastrula	Regressing gastrula	Prism	Pluteus
12 hours	5	5	90	0	0
18 hours	1	24	9	65	1
24 hours	2	22	7	59	10
36 hours	3	0	0	2	95

* A minimum of 200 embryos was counted for each age.

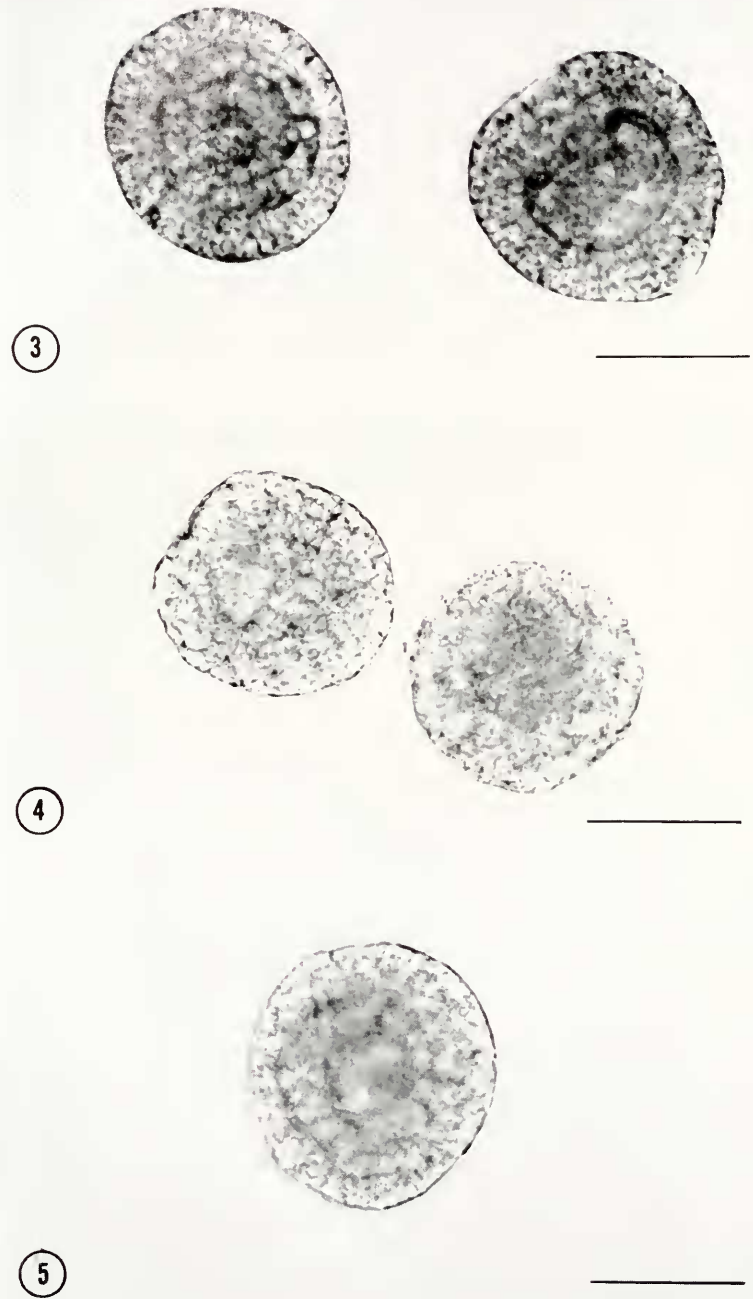
in comparison with controls. Further, normal, young plutei treated with HBO at 48 hours post-fertilization age were observed at 72 hours to swim much more slowly than did controls. These latter observations may possibly reflect a general depression of metabolic activity caused by HBO.

Controls

Embryos reared in 1 AA pure oxygen or in 1 AA pure oxygen plus 2 AA nitrogen did not differ in morphology or in developmental rate from embryos reared in air at normobaric pressure (see Figs. 11 and 12).

DISCUSSION

The patterns of effects on development of HBO are unique in the two species which we have studied. In contrast with toxic agents which produce death, differential inhibition, differential tolerance, conditioning or recovery depending upon the concentration and duration of the exposure (Child, 1941), hyperbaroxia appears



FIGURES 3-5.

to block development totally for extended periods of time and yet permits total or nearly total reversibility in its effects. The reversibility in *Tubularia* development is striking with total blockage of differentiation for a period of as much as five days followed by the formation of completely normal actinula larvae (Miller *et al.* 1969). In *Arbacia*, exposures which arrested gastrulation and induced apparent dedifferentiation and regression of the developing mesoderm and endoderm never-the-less were followed by complete recovery of normal morphology in more than 90% of the embryos. There were virtually no inhibited larvae with reduced oral lobes such as are characteristic of embryos exposed to KCN, CuSO₄, LiCl and many other toxic agents (*cf.* Fig. 47, page 199, Child, 1941).

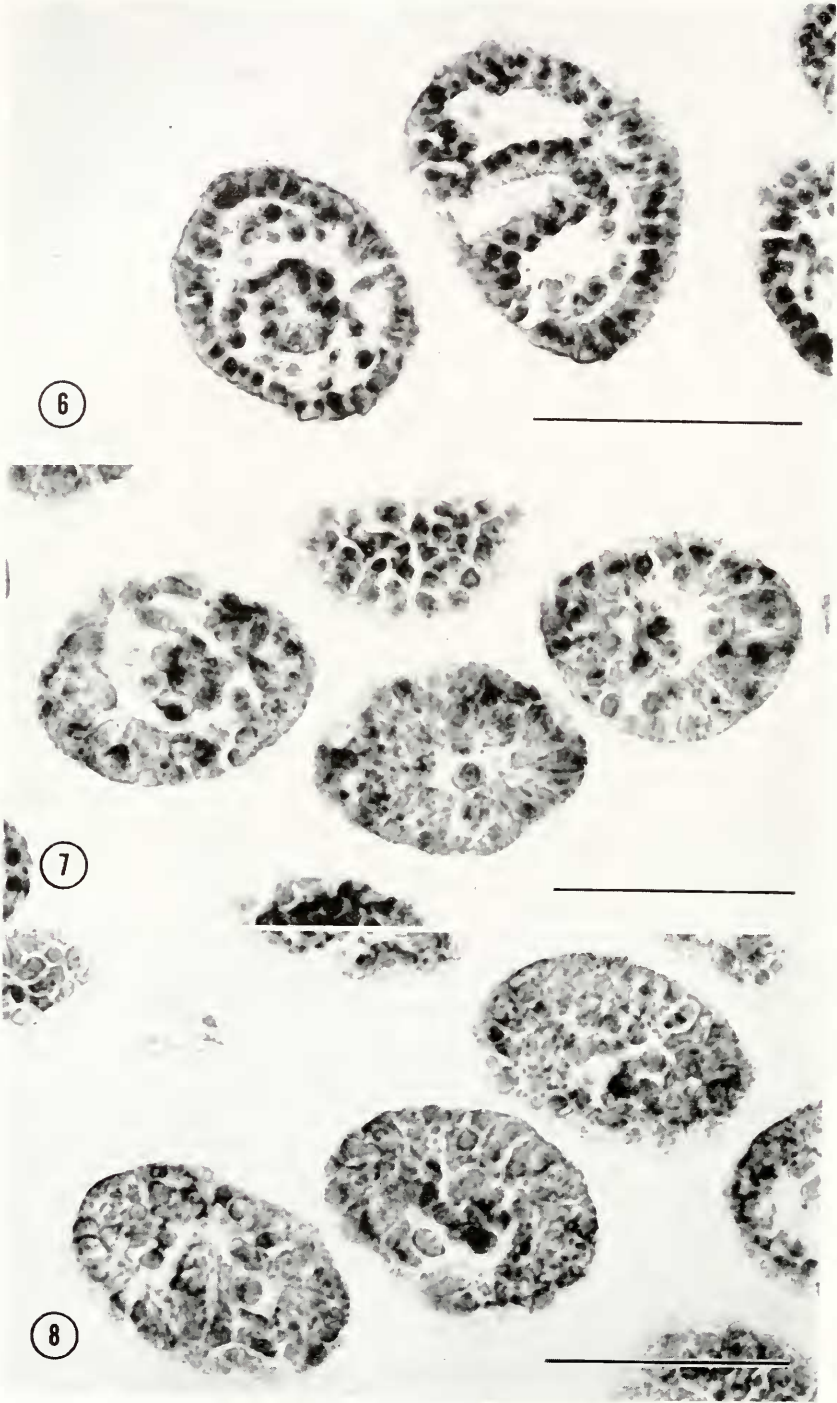
This suggests that the blockage which was produced by HBO did not induce the production of toxic substances which altered any future differentiation. Instead, it appears that, although blocked temporarily, their biochemical systems were not permanently injured, and could redifferentiate as soon as the excess oxygen was removed. These findings are consistent with the concept that the fundamental effect of hyperbaric oxygen is to oxidize enzymes which are active in the reduced state but are inactivated when in the oxidized condition (Haugaard, 1968).

Hyperbaric oxygen has been shown to inactivate sulfhydryl-containing enzymes including succinic dehydrogenase (Haugaard, 1968; Davies and Davies, 1965). It is significant that this enzymatic activity has been shown to be high during gastrulation in the sea urchin (Gustafson and Hasselberg, 1951). A second enzymatic activity which is high in activity during gastrulation in the sea urchin is that of cathepsin II, which is low in activity up to the mesenchyme blastula stage, but which undergoes a sharp rise after this stage (Gustafson and Hasselberg, 1951). This enzyme is considered sulfhydryl-dependent (Fruton, Irving and Bergmann, 1941) and is known to be inhibited by HBO (Davies and Davies, 1965; Rosenbaum, 1960). Furthermore, it has been demonstrated that the flavoprotein, diaphorase, is inactivated by oxidation (Williams, 1965). Diaphorase-dependent dehydrogenase activities, such as those of glucose-6-phosphate dehydrogenase and malic dehydrogenase, might thus be indirectly inhibited under HBO. In this regard, it is significant that glucose-6-phosphate dehydrogenase has been shown in *Paracentrotus* to be maximally active immediately preceding, and through the first half of gastrulation (Backstrom, 1959); malic dehydrogenase activity rises during the mesenchyme blastula stage, gastrulation and later stages, paralleling the rise in succinic dehydrogenase activity (Gustafson and Hasselberg, 1951). The blockage of such enzymatic activities during development would be expected to disrupt development during the stages at which they are highly active; this correlates well with our observations of blockage within gastrula stage of HBO-treated embryos. Direct biochemical analysis of enzymatic activity during the course of HBO treatment of sea urchin embryos is in progress.

FIGURE 3. Control gastrulae, 36 hours post-fertilization age. Embryos were reared in air at normobaric pressure at 16° C; whole mount. Scale on all photomicrographs equals 50 μ .

FIGURE 4. Embryos reared in HBO at 3 AA, 16° C, for 36 hours: "regressing gastrula" stage. Archenteron structure is indistinguishable in these embryos. Compare with control embryos shown in Figure 3; whole mount.

FIGURE 5. Embryo reared in HBO at 3 AA, 16° C, for 48 hours. Morphology is similar to the regressing gastrulae shown in Figure 4; no additional morphological changes are observed with the 12 hours of additional exposure to HBO; whole mount.



FIGURES 6-8.

Our studies correlate well with those of Rosenbaum (1960) who reported arrest in the gastrula stage in frog embryos treated with HBO, and with those of Malamed (1957) who observed similar blockage in *Rana pipiens* embryos following incubation of eggs in a medium through which oxygen was bubbled. No studies have been made of the protection afforded embryos from the effects of HBO by BAL, cysteine, or glutathione (Wittner and Rosenbaum, 1958a, 1958b).

Series 1

Even though the "regressing gastrula" embryos which were observed at both 36 and 48 hours were indistinguishable morphologically from each other (compare Figs. 4 and 5, and Figs. 7 and 8), the 48-hour embryos were much more severely inhibited, as evaluated in terms of the time necessary to complete development through the pluteus stage (see Figs. 1 and 2). The population of embryos incubated in HBO for 48 hours required approximately twice the time, following removal from the chamber, to complete development through the pluteus stage as did those which were exposed to HBO for 36 hours. From observations of whole mounts, it appeared that the internal rearrangement of cells during recovery of the regressed gastrulae might not be dissimilar to that observed by Giudice and Mutolo (1970) in sea urchin embryos reaggregating from dissociated cells. These authors have shown that the intestine which develops from reaggregated spheres of cells does not form by invagination, as in normal development, but by the internal rearrangement of cells about a space destined to become the intestinal lumen. However, a detailed histological study of the reversal of inhibition seen in the present study remains to be performed.

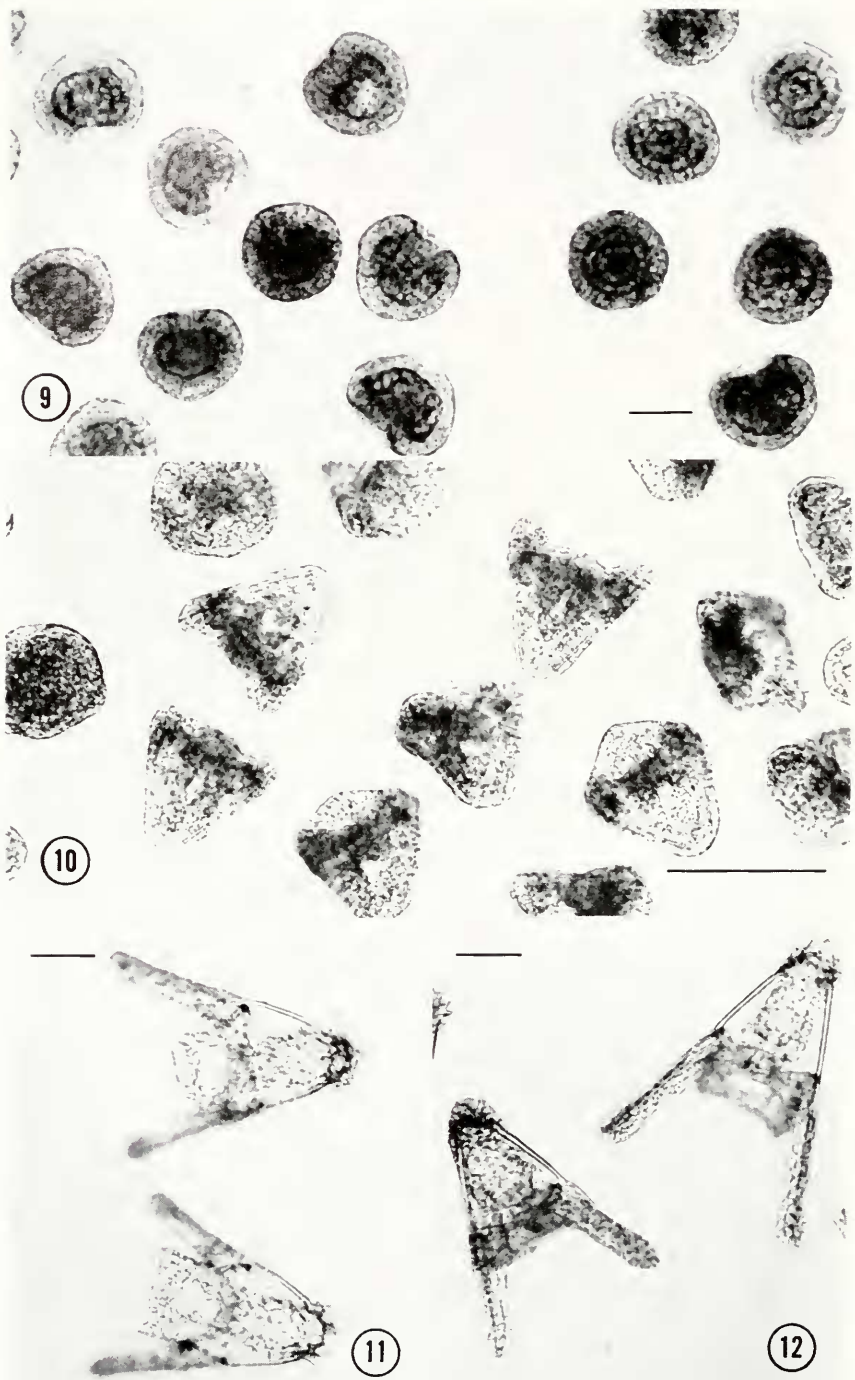
Series 2 and 3

It is instructive to examine the results of Series 2 and 3 in terms of assessing the time at which HBO may exert its inhibitory effect. Hyperbaric oxygen exerted no evident inhibitory effect upon embryos exposed continuously for 12 hours following fertilization; by 24 hours, however, an inhibition to development was manifested by the delay in the time of pluteus formation. The inhibition was observed to be more severe following 36 or 48 hours and was accompanied by the characteristic morphology of the "regressing gastrula." There would appear, therefore, to be no residual inhibition of processes regulating pluteus formation from only 12 hours of exposure to HBO. Alternatively, those processes essential to normal gastrulation and pluteus formation and susceptible to HBO inhibition may not be operative at such early stages. These processes may differentiate at

FIGURE 6. Histological section of control gastrulae reared in air at normobaric pressure for 36 hours at 16° C. Note the typically prominent archenteron structure and associated mesoderm; 7-micron section, hematoxylin and eosin stain.

FIGURE 7. Histological section of "regressing gastrulae." Fertilized eggs were exposed continuously to 3 AA oxygen for 36 hours at 16° C. Note the absence of an organized archenteron and the "blastula-like" appearance of the embryos; cf., Figure 6; 7-micron section, hematoxylin and eosin stain.

FIGURE 8. Histological section of "regressing gastrulae." Fertilized eggs were exposed continuously to 3 AA oxygen for 48 hours at 16° C. Note morphology similar to that in Figure 7 above; 7-micron section, hematoxylin and eosin stain.



FIGURES 9-12.

later stages, and be inhibited by exposure to HBO later in development, in the mesenchyme blastula stage. It should be noted, however, that HBO may possibly exert a generalized depressive effect upon the metabolism of plutei; this is perhaps reflected in the slow swimming movements of morphologically normal plutei which were evident after prolonged exposure to HBO.

From Series 3 it may be inferred that embryos which have reached late gastrula (36 hr) prior to exposure to HBO have already been "programmed" for pluteus formation, and HBO at 3 AA exerts no effect upon this stage of morphogenesis. No information, however, is available in this system concerning possible delays in the action of HBO, as is seen in mammals (Haugaard, 1968). The possibility of such delays in effective inhibition must be investigated before a valid timetable of inhibition may be established.

Although no detailed comparative study of the initial cleavage patterns of control and HBO-treated embryos was made, no obvious effect of HBO upon cleavage pattern was noted in the present study, and both HBO-treated and control embryos developed synchronously until the time of gastrulation. These findings are in accord with those of Rosenbaum and Wittner (1960) who found in *Echinarachnius* that 4-hour exposures to oxygen at 3 to 4 atmospheres induced no anomalies of cleavage or development within the first 8 hours of development. Higher pressures of oxygen (7 to 8 AA) did inhibit early cleavage; later embryonic development under hyperbaric conditions was not studied. It would be of interest to determine whether or not the delay period in inhibition in our experiments would be shortened by using oxygen pressures higher than 3 AA.

In this species, as in our previous experiments with *Tubularia*, (Miller *et al.*, 1969) hyperbaric oxygen has proven to be a reversible inhibitor of development; it is hoped that our findings may stimulate the use of HBO in a variety of systems as a tool in studies of morphogenesis.

SUMMARY

The effects of hyperbaric oxygen (HBO) upon the embryonic development of *Arbacia* were studied from fertilization to the time of formation of the pluteus larva. Fertilized eggs were incubated in sea water at 16° C in air or in 3 atmospheres absolute (AA) pure oxygen in a hyperbaric chamber. Pressure control experiments using 1 atmosphere oxygen and 2 atmospheres nitrogen demonstrated that the changes observed were caused by elevated pressure of oxygen, and not merely to high ambient pressure. Animals exposed continuously to hyperbaric oxygen for 48 hours were arrested in the gastrula stage; the archenteron

FIGURE 9. Normal embryos which have developed in air at 16° C for 36 hours, gastrula stage. Compare morphology with that of the inhibited gastrulae shown in Figure 4; whole mount.

FIGURE 10. Normal embryos which have developed in air at 16° C for 48 hours, young plutei. Compare with embryo which has developed for the same period of time at the same temperature, but under 3 AA oxygen, shown in Figure 5; whole mount.

FIGURE 11. Pluteus larvae reared at 16° C under normobaric conditions in air for 72 hours; whole mount.

FIGURE 12. Pluteus larvae reared at 16° C under 3 AA pressure (1 atmosphere oxygen, 2 atmospheres nitrogen) for 72 hours. Plutei are indistinguishable morphologically from embryos reared under normobaric conditions (*cf.* Fig. 11); whole mount.

was observed to form at 30–32 hours, but to regress, resulting in an unorganized mass of cells within the blastocoel. If removed from HBO at 48 hours, over 90% of these inhibited embryos proceeded to form normal prisms and plutei within 144 hours following removal. In respect to reversibility, therefore, HBO differs significantly from many chemical inhibitors of differentiation.

Embryos reared in HBO following development in air for the first 12 hours after fertilization failed to form plutei by 72 hours, whereas controls did so within 48 hours. Embryos in which exposure to HBO was delayed 18 or 24 hours were delayed in reaching the prism or pluteus stage, and showed evidence of disaggregation within 72 hours post-fertilization. Embryos exposed to HBO following 36 hours of development escaped both the inhibitory and lethal effects of HBO and proceeded to form normal plutei by 48 hours. The inactivation by HBO of sulfhydryl-containing enzymes which normally are maximally active during gastrulation in the sea urchin may contribute to the failure of embryos treated with hyperbaric oxygen to complete gastrulation.

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